

Allergenicity and immunogenicity of Basidiomycetes

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Species selected from six families of the class Basidiomycetes were evaluated for allergenicity in atopic and nonatopic individuals and for immunogenicity and antigenic cross-reactivity in experimental animals. Between 42% and 68% of atopic asthmatics demonstrated positive Type I wheal-and-flare skin reactivity to basidiomycete metabolic and somatic antigens. Sixty-four percent of skin test-positive atopic asthmatics exhibited positive RAST to a basidiomycete metabolic antigen and 50% were positive to somatic antigen. Negative RAST results were obtained in all non-atopic control sera. Only an occasional individual demonstrated positive serum anti-basidiomycete precipitins by counterimmunoelectrophoresis. All basidiomycete species studied were highly immunogenic in the rabbit and most appeared to contain an electrophoretically heterogeneous group of antigens with predominant anodal mobility. Ouchterlony double-diffusion analysis employing hyperimmune rabbit antisera indicated the presence of shared antigens among all basidiomycete species with the exception of Pleurotus. Rabbit antibasidiomycete sera did not cross-react with antigens of several common species of the Fungi imperfecti. Results indicate that many atopic asthmatics of the Gulf South area demonstrate IgE-mediated hypersensitivity to basidiomycete antigens as evidenced by positive wheal-and-flare skin reactivity and/or RAST. Basidiomycetes are also immunogenic in the rabbit and possess antigens that do not cross-react with those of certain Fungi imperfecti.

It has long been established that fungal spores are present in the atmosphere in concentrations considerably in excess of pollen grains. Many of the *Fungi imperfecti* are known to induce respiratory allergic reactions in sensitized individuals, as demonstrated by the presence of wheal-and-flare skin reactivity and provocative inhalation challenge testing.¹⁻⁴ Most studies of fungi as aeroallergens have, however, been limited to the *Fungi imperfecti* and little information is available with regard to allergenicity and immunogenicity of other fungal classes.

The Basidiomycetes class, the most advanced of all fungi, has between 20,000 and 25,000 species, and it has been recently shown that members of this class are present in high atmospheric concentrations in certain geographic areas.⁵⁻⁷ In the United States, however, little is known with regard to atmospheric concentrations and allergenicity of Basidiomycetes. In this study species selected are evaluated

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for allergenicity in atopic and nonatopic individuals and for immunogenicity and antigenic cross-reactivity in experimental animals.

MATERIALS AND METHODS

Basidiomycete cultures

The following representative cross-section of easily culturable basidiomycete families were obtained from the American Type Culture Collection: *Tilletiopsis minor* (ATCC 10764), *Dacrymyces deliquescens* (ATCC 13292), *Stereum frustulatum* (ATCC 12682), *Pleurotus ostreatus* (ATCC 9415), *Hypholoma sublateritium* (ATCC 9413), *Cantharellus cibarius* (CC), and *Coprinus comatus* (ATCC 12640). Organisms were grown at room temperature in Czapek-Dox broth (Difco Labs., Detroit, Mich.) for 15 days on shaker incubators and stock cultures maintained in broth at room temperature with repeated subcultures.

Antigens

For metabolic basidiomycete antigens 15-day culture growths were centrifuged and the supernatant was decanted and dialyzed against distilled water for 72 hr, followed by lyophilization. Dialyzed, lyophilized supernatant was designated as "basidiomycete metabolic antigen." The remaining mold mat was resuspended 1:10 w/v in 0.018M NaCl, disrupted in a Waring blender, and allowed to extract overnight (16 hr) at 4° C. After centrifugation the supernatant was filtered with Whatman filter paper, dialyzed against distilled water for 72 hr, and lyophilized for use as "basidiomycete somatic antigen." Lyophilized somatic and metabolic antigens were reconstituted in 0.5% phenolized saline in concentrations of 1.0, 0.1, and 0.01 mg/ml followed by Millipore filtration for use in skin testing. For gel diffusion studies, lyophilized antigen was reconstituted in veronal buffer in concentrations of 20 mg/ml.

Fungi imperfecti antigens for skin testing were prepared from four species: *Hormodendrum hordei* (ATCC 12092), *Penicillium notatum* (ATCC 9178), *Alternaria tenuis* (Tulane Infectious Disease Isolate), and *Aspergillus fumigatus* (Tulane Infectious Disease Isolate). The *Fungi imperfecti* employed for gel diffusion analysis consisted of somatic antigens of these species plus *Fusarium solani* (ATCC 12823). Organisms were grown in synthetic medium followed by centrifugation, extraction of the mold mat, as in the case of basidiomycete antigens, and lyophilization. Antigens were reconstituted in 0.5% phenolized saline (1.0, 0.1, and 0.01 mg/ml) for skin testing and in veronal buffer (20.0 mg/ml) for gel double-diffusion studies.

Immunogenicity in rabbits

Antisera. Thirty-five 3.0-3.5 kg white New Zealand rabbits were divided into subgroups of 5 each. Animals were immunized with 10.0 mg of crude unextracted lyophilized 15-day growth of each basidiomycete species in 0.4 ml of Freund's complete adjuvant (Difco Labs.) via the toe pad route followed by 6 subcutaneous monthly "booster" injections of 10.0 mg of crude antigen in saline solution. Animals were bled at monthly intervals for 6 mo. Five and 6 mo sera, frozen without preservative, from each subgroup were pooled for analysis by Ouchterlony double diffusion.

Gel diffusion studies employing rabbit hyperimmune sera. Immunoelectrophoresis was performed on 103 by 83 mm Kodak lantern slides layered with 15.0 ml of 0.82% agarose (Sigma Chemical Co., St. Louis, Mo.) in veronal buffer, pH 8.2, with a previously described semimacromethod.⁸ Antigens solubilized in veronal buffer (7.0 μ l) were subjected to electrophoresis with a potential gradient through the gel of 5.6 volts/linear cm of agar $\times$ 90 min. Following electrophoresis, troughs were filled with 0.25 ml of rabbit serum prepared against each respective basidiomycete antigen. Diffusion was carried out for 48 hr at room temperature in a humidified chamber followed by washing for 72 hr, drying, and staining with Light Green S.F.

Ouchterlony double-diffusion analysis was performed with 0.82% agarose and pooled rabbit antisera against each basidiomycete species.

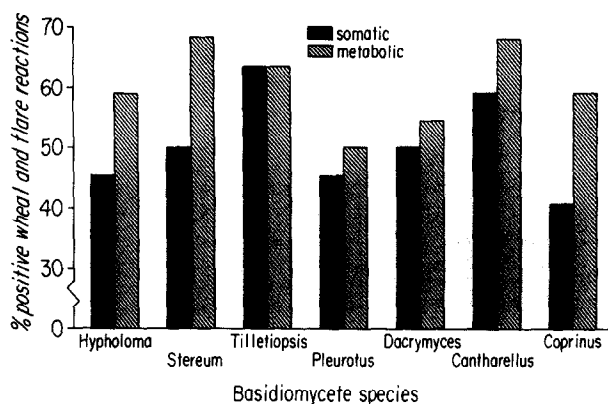


FIG. 1. Incidence of positive wheal-and-flare skin reactivity of 25 atopic asthmatics to somatic and metabolic antigens of seven basidiomycete species.

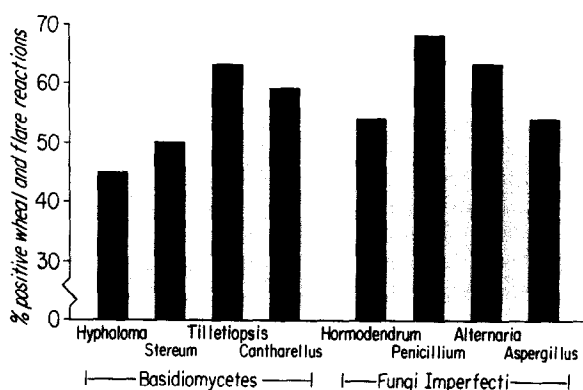


FIG. 2. Incidence of wheal-and-flare skin reactivity to somatic antigens (0.1 mg/ml) in 0.5% phenolized saline of 4 common **Fungi imperfecti** (*H. hordei*, *A. fumigatus*, *P. notatum*, and *A. tenuis*) and 4 basidiomycetes in 25 atopic asthmatics.

Immune response in man

Skin tests. Groups of 25 atopic and 25 nonatopic subjects were chosen for purposes of skin testing with metabolic and somatic antigens. Atopic subjects were selected from the Charity Hospital Allergy Clinic population on the basis of personal history of bronchial asthma and presence of a broad pattern of wheal-and-flare skin reactivity to a battery of common local inhalant allergens as previously described.⁹ These subjects were predominantly black women ranging from 19 to 52 yr of age.

Nonatopic subjects consisted of predominantly white medical students and laboratory technician volunteers ranging from 22 to 38 yr of age and having no personal or past history of allergic respiratory disease and negative wheal-and-flare skin reactivity to common local inhalant allergens. All study subjects were long-term residents of the Gulf South area.

Prick tests were performed on the volar forearm with freshly prepared Millipore filtered metabolic and somatic antigens in 1 mg/ml concentration. Intradermal tests were performed with a tuberculin syringe and 27-gauge needle (0.02 ml) containing 0.01, 0.1, and 1.0 mg of metabolic or somatic antigen per milliliter (approximately 0.2, 2.0, and 20.0 μ g absolute quantity per test). Reactions were read at 20 min and recorded in terms of millimeters of

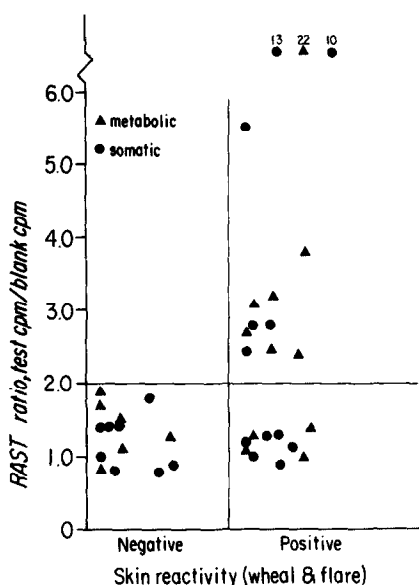


FIG. 3. Comparison between RAST ratios (test cpm/blank cpm) and wheal-and-flare skin reactivity to *Cantharellus cibarius* somatic antigen (0.1 mg/ml) in selected atopic asthmatic subjects (17 comparisons for metabolic antigen and 20 for somatic antigen).

edema and erythema. Subjects were not observed for Arthus or delayed skin reactions. Those reacting to a particular antigen by the prick method were not tested with that antigen by the intradermal method.

RAST. Filter paper discs were activated with cyanogen bromide by the method of Ceska, Eriksson, and Varga.¹⁰ *Cantharellus cibarius* was chosen as the basidiomycete antigen for RAST because it induced large wheal-and-flare skin reactions in many atopic asthmatic subjects. To couple the somatic or metabolic *Cantharellus* antigen, a sufficient volume of a 20 mg/ml solution of antigen in borate buffer, pH 8.0, was added to activated discs so that the discs were covered with antigen solution. Discs were incubated at room temperature for 6 hr on a rotator, washed three times with 1 M ethanolamine, pH 8.0, and stored overnight in ethanolamine at 4° C followed by washing in assay buffer (500 ml of 0.2 M PO₄ buffer, pH 7.5; 500 ml of 1.8% NaCl (w/v); 10 ml of 5% NaN₃; 5 ml of Tween 20, 2 gm of BSA).

One coupled disc and 50 μ l of test serum were incubated on a rotator for 3 hr at room temperature. After washing three times with physiologic saline, 0.1 ml of radioiodinated anti-IgE (¹²⁵I-anti-IgE, Pharmacia Labs., Inc.) was added, followed by incubation at room temperature on the rotator overnight. Discs were washed three times with saline, transferred to a counting vial, and counted in a Beckman Biogamma counter. Test counts were compared with counts obtained with discs treated as above but without addition of serum. Following the rationale of Wide, Bennich, and Johansson,¹¹ a ratio of test:blank ≥ 2 was considered as a positive test. All tests were performed in duplicate. A positive serum (C.A.) was used as a positive control in different test runs. A test of 6 blank discs gave, for metabolic antigen, a mean of 667.3 cpm with a standard deviation of 56.7 and, for the somatic antigen, a mean of 548.3 cpm with a standard deviation of 23.4. The positive control serum gave a mean test:blank ratio of 4.73 with a standard deviation of 0.95.

Serum precipitins. Assays were performed for precipitins against somatic antigens of *Cantharellus cibarius*, *Dacrymyces deliquescens*, *Stereum frustulatum*, *Hypholoma sublateritium*, *Pleurotus ostreatus* and *Tilletiopsis minor* (20.0 mg/ml) by counterimmunoelectrophoresis (CIE) in 0.82% ionagar No. 2 and veronal buffer.¹² Electrophoresis was carried out for 60 min at constant voltage (5.4 v/linear cm of agar) in veronal buffer at pH 8.2.

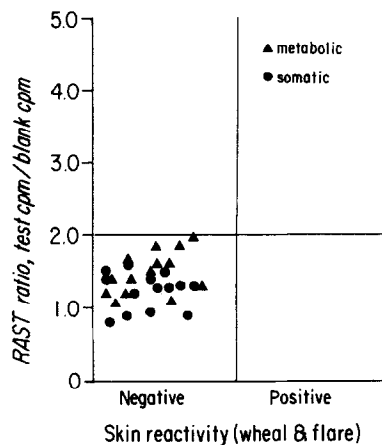


FIG. 4. Comparison between RAST ratios (test cpm/blank cpm) and wheal-and-flare skin reactivity to *Cantharellus cibarius* somatic antigen in 14 nonatopic controls.

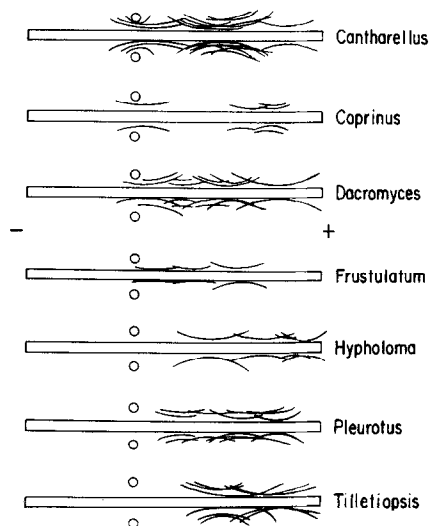


FIG. 5. Immunoelectrophoresis of basidiomycete somatic antigens against pooled specific rabbit hyperimmune serum (20.0 mg/ml in antigen wells and 0.25 ml specific pooled rabbit serum in central troughs).

RESULTS

Skin tests

The incidence of wheal-and-flare skin reactivity to metabolic and somatic antigens of seven basidiomycete species in atopic asthmatic subjects is listed in Fig. 1. Between 42% and 68% of this group demonstrated positive skin reactivity to the 7 basidiomycete species employed by a combination of prick and intradermal tests (0.2 to 2.0 μ g in 0.02 ml volume) with a slightly higher incidence being noted with metabolic antigens. The 1.0 mg/ml skin test dosage (20.0 μ g in 0.02 ml volume) produced positive reactions in several control

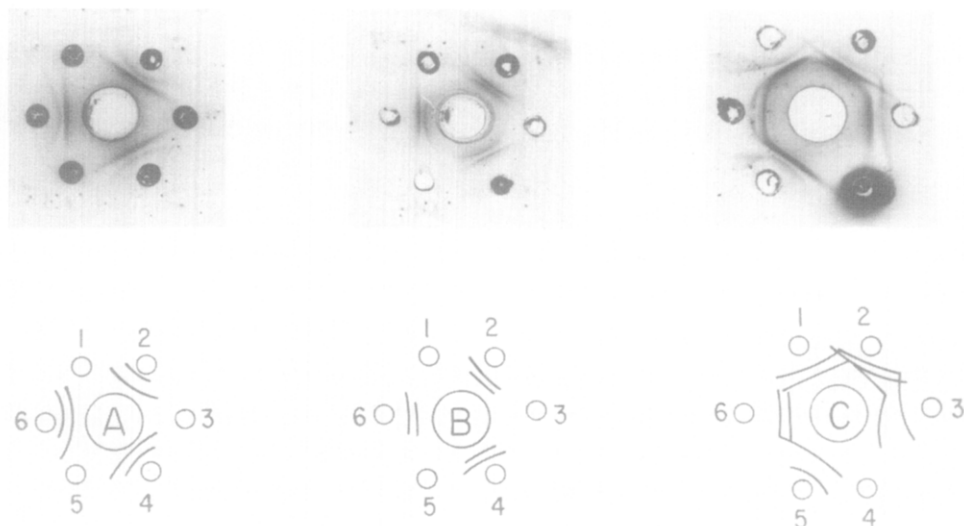


FIG. 6. Ouchterlony double-diffusion analyses of: **A**, rabbit anti-*Cantharellus* serum; **B**, rabbit anti-*Dacrymyces* serum; and **C**, rabbit anti-*Cantharellus* serum (center wells). Peripheral wells 1, 3, and 5 in templates **A** and **B** contain lyophilized somatic antigens of *Fusarium solani*, *Penicillium* sp., and *Hormodendrum hordei*, respectively (20.0 mg/ml reconstituted in veronal buffer). Wells 2, 4, and 6 contain *Cantharellus* somatic antigen (template **A**) and *Dacrymyces* somatic antigen (template **B**). Peripheral wells (in template **C**) contain the following somatic antigens: 1 and 3, *Dacrymyces*; 2, *Cantharellus*; 4, *Pleurotus*; 5, *Tilletiopsis*; and 6, *Hypholoma*.

subjects and results at this dose level were not used for data analysis. The incidence of wheal-and-flare skin reactivity to 4 common species of the *Fungi imperfecti* was also high in these subjects, ranging between 53% and 68% for the 4 species employed (Fig. 2).

Serum precipitins

One atopic and 1 nonatopic subject demonstrated reproducible precipitins against *Dacrymyces*, 1 nonatopic subject was positive to *Hypholoma*, and 1 atopic patient revealed a faintly positive precipitin band against *Tilletiopsis*. All other sera revealed negative precipitin reactions on duplicate determinations against all basidiomycete antigens with the exception of *Pleurotus ostreatus*, which developed clear-cut thick precipitin bands with all sera tested. We have previously noted this type of uniformly positive nonabsorbable precipitin reaction (precipitins present in ammonium sulfate-precipitated serum fractions and IgG-rich fractions obtained by gel filtration) with a *Puccinia coronata* (grass smut) basidiomycete antigen. This batch of *Puccinia* had been harvested directly from the plant under unsterile conditions and was likely contaminated with bacterial antigens.¹³

Rast

Results of RAST assays employing *Cantharellus cibarius* metabolic and somatic antigens are illustrated in Fig. 3 and 4. All nonatopic individuals

demonstrated negative wheal-and-flare skin reactivity to *Cantharellus* somatic and metabolic antigens and negative RAST tests (based on arbitrarily chosen value of < 2.0 test cpm/blank cpm ratios as a "negative" test). With *Cantharellus* metabolic antigen 7 of 11 skin test-positive atopic individuals (64%) exhibited positive RAST tests and six of 12 (50%) were positive with somatic antigen. All atopic individuals with negative wheal-and-flare skin reactivity to *Cantharellus* antigens also exhibited RAST ratios below 2.0, indicating excellent correlation between negative wheal-and-flare skin reactions and negative RAST assays.

Immunogenicity in rabbits

Fig. 5 illustrates results of immunoelectrophoretic analyses employing pooled hyperimmune rabbit sera and somatic antigens of seven basidiomycete species. All species were immunogenic in the rabbit and most appeared to contain an electrophoretically heterogeneous group of antigens with predominant anodal mobility. Certain species such as *Cantharellus cibarius* and *Dacrymyces deliquescens* were highly immunogenic when compared with others such as *Coprinus comatus* and *Hypoholoma sublateralitium* which demonstrated only three to four major antigens against specific pooled hyperimmune serum.

Hyperimmune sera prepared against all basidiomycete species were reacted against somatic antigens of each respective species on multiple Ouchterlony double-diffusion analyses and results indicated the presence of shared antigens among all seven species studied with the exception of *Pleurotus*, which reacted only against specific rabbit anti-*Pleurotus* serum. Rabbit antibasidiomycete sera did not cross-react with antigens of several common species of the *Fungi imperfecti* on gel diffusion analysis (Fig. 6), but the high incidence of wheal-and-flare skin reactions to allergenic extracts of *Fungi imperfecti* in basidiomycete-sensitive asthmatic subjects suggested the possible presence of common allergens among members of those classes.

DISCUSSION

Because the identification of basidiospores is difficult and information from culture sampling often unreliable, we elected to obtain information regarding the role of basidiospores as aeroallergens in our atopic asthmatic and "normal" populations. The present report indicates that many atopic asthmatic persons demonstrate IgE-mediated hypersensitivity to basidiomycete antigens as evidenced by positive immediate wheal-and-flare skin reactivity and/or RAST. Nonatopic control subjects uniformly demonstrated negative immediate skin reactions and RASTs to basidiomycete antigens (based on an arbitrarily chosen value of < 2.0 test cpm/blank cpm ratio as a negative RAST). Atopic asthmatic patients with negative basidiomycete skin reactivity also demonstrated negative RAST assays but several atopic subjects exhibiting positive basidiomycete wheal-and-flare skin tests demonstrated negative RAST. This lack of perfect correlation between RAST and immediate skin reactivity is likely due in part to our use of crude metabolic and somatic antigens and a lesser sensitivity of RAST when compared with the wheal-and-flare skin test.¹⁴ Our results further

indicate that basidiomycetes are immunogenic in the rabbit and possess antigens that do not cross-react with those of certain *Fungi imperfecti*.

Awareness of the potential importance of basidiospores as aeroallergens has recently become more apparent. Gregory and Hirst⁵ called attention to their steady high atmospheric concentrations between June 1 and Sept 30 in Harpenden, England, and these observations have been confirmed by others in Scotland and Wales.^{7, 15}

In Cardiff, during the years 1962-1966, basidiospores constituted 29% of the fungal aerospore catch as compared to 25% for *Cladosporium*, one of the most prevalent of the *Fungi imperfecti*.^{7, 16} Other investigators have also reported that basidiospores and ascospores comprised 54% of the total spore catch at Liverpool and 29% at London.¹⁷ Although the 1964 summary of airborne mold surveys in the United States did not mention basidiospores as part of the total airborne spore population,¹⁸ it is reasonable to assume that they are prevalent in this country. Suggestive evidence for such a high prevalence rate is provided by a recent study of Kramer and co-workers,¹⁹ who performed daily estimates of aerospores in Manhattan, Kan., and noted that 24.3% of all spores were basidiospores, an incidence second only to that of *Cladosporium*. In studies of New Orleans asthma we have also noted that asthma "epidemics" of considerable magnitude during the late summer and fall months were associated with high total spore and basidiospore catches.^{20, 21} Thus it is evident that in some parts of the world basidiospores form an important part of the total airborne spore concentration. Previous failure to detect their presence has likely been due in part to the aerometric sampling methods employed. The gravitational method is known to be inefficient in trapping small spores and the similar morphologic characteristics of basidiospores and many other small spores makes their identification difficult by light microscopy. The Petri dish exposure method also detects only a small fraction of the atmospheric fungal load due to such factors as competition between fast and slow growing species, duration of exposure, and type of culture medium employed.³

Although these failures to detect basidiomycetes as major parts of the airborne spore catch have delayed studies of their potential role as aeroallergens in man, positive responses to several basidiospores by both skin test (1:1,000 w/v extracts) and inhalation challenge have been reported.^{22, 23} In these studies the frequency of positive wheal-and-flare skin reactivity to basidiospores in asthmatic patients was actually similar to that obtained with certain common clinically important species of the *Fungi imperfecti*. In another study of woodlands as sources of basidiospores in Cardiff, Wales, Adams, Hyde, and Williams¹⁶ made preliminary conclusions, based on known seasonal patterns and quantitative aerometric sampling data, that basidiospores were likely both antigenic and capable of producing epidemics of allergic airway disease due to their regular seasonal occurrence in high concentrations.

Our current findings now provide data that basidiospores may serve as important aeroallergens in atopic asthmatics who reside in the United States Gulf South area, based on our detection of a high degree of wheal-and-flare skin reactivity and positive RAST assays in many of these patients. Our previous

detection of particles having the morphology of basidiospores in high concentrations during certain late summer and fall epidemics of New Orleans asthma together with their known allergenicity in this population further suggest that they may provide an important antigenic stimulus in production of these outbreaks. Provocative inhalation challenge tests in selected basidiospore-sensitive subjects should provide further direct evidence for their ability to serve as inhalant allergens and such studies are in progress.

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